PHOTOCHEMICAL SPECTRAL ANALYSIS OF NEURAL TUBE FORMATION [1]

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Introduction

In recent years the analysis of morphogenesis has been concerned with the study of the chemical nature of substances which play a decisive role in developmental phenomena. In the field of neural induction the usual procedure used is that of extraction of the active material with solvents specific for a particular group of compounds; the degree of substitution is tested by implantation of the extracted material. This approach has failed to yield conclusive results because large quantities of tissue known to contain the normal inductor are not available. Investigators have been unable to determine whether induction resulting from implanted substances is the result of the direct action of the substance on the tissue or of a substance released in the reacting tissue. This technique is also subject to the criticism that two chemical substances may not necessarily be identical because they produce the same histological or morphological changes. It is a well-known fact that histological changes produced in the vagina and uterus by a number of artificial estrogens are identical with those produced by the natural estrogens (McKenzie, 1941).

The investigation of active substances need not be restricted to attempts to isolate them, although isolation and synthesis is the ultimate goal. If the action of a developmental substance is inhibited by a specific agent, a preliminary identification will have been made and it will be certain that the substance inactivated is operative in the organism. The use of chemical poisons has demonstrated the importance of this technique in the field of cellular oxidation. The technique of the photochemical inactivation of substances involved in the developmental processes has enabled the investigator to study the chemical nature of this material during its action in normal development.

The classical experiments of Warburg (1927) in the identification of the respiratory enzyme by absorption spectrophotometry show the importance of the technique of photochemical inactivation. This method involves the irradiation of a biological system with monochromatic radiation and the consequent inactivation of a chemical substance in the system. Absorption is measured indirectly in terms of a physiological or morphological change produced in the biological system. Warburg measured absorption by determining the change in oxygen consumption of yeast cells in the presence of carbon monoxide following irradiation with monochromatic light. In the present investigation absorption is measured in terms of the amount of energy required to inhibit the folding process in neural tube forma-

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tion. By this method an indirect photochemical absorption spectrum of the material involved in a biological activity can be obtained. By comparing this spectrum with the absorption spectra of chemical compounds, information can be ascertained concerning the chemical nature of the material.

A preliminary exploration showed that ultraviolet radiation would not inhibit the transformation of gastrula ectoderm into neural plate unless extremely large doses were used and the cells severely altered or killed. However, the folding process of neural tube formation was inhibited by very weak doses of ultraviolet light with little effect on the embryo in other ways. The neural plate merely continued to develop as a plate. The effect was uniform enough to compare quantitatively the effects of different wave-lengths. Consequently, an attempt was made to identify by its absorption spectrum a substance which is apparently of decisive importance in the process of neural tube formation.

Since most of the work concerned with neural tube formation has been done in Amphibia, it might be expected that this material would offer more advantages than any other. This is not the case. Amphibian embryos possess yolk granules and pigment which absorb and scatter incident radiation. Consequently, the photochemical efficiency curve obtained for inhibition of the folding process in Amphibia would not give a true measure of absorption. For this reason and because an abundant source of avian material was available, it was decided to use chick embryos in this investigation.

The author wishes to thank Dr. Daniel Mazia under whose direction the study was made. Grateful acknowledgment is made to Dr. F. M. Uber who permitted the author to make the thermopile measurements in his laboratory and to Dr. L. J. Stadler for use of the monochromator.

Materials and Methods

The material for this study consisted of the eggs of two breeds of the domestic fowl, the White Leghorn and the New Hampshire Red. In order to secure uniform results, all the eggs were obtained from two pens of hens, one of New Hampshire Reds and the other of White Leghorns. The University of Missouri poultry farm was the source of this material.

Each egg was incubated and the position of the blastoderm determined by candling. The egg was then placed in a Syracuse watch glass which contained modeling clay to hold the egg in place. Only sterile equipment was used in these experiments. The surface of the egg was sterilized with a piece of cotton which previously had been soaked in 70 per cent alcohol. An opening of 7 to 9 sq. mm. was cut in the egg shell by means of a small saw. This revealed the blastoderm through the shell membrane. After removal of the shell membrane with forceps, a sterile .9 per cent salt solution was used to float the embryo to the level of the surface of the egg shell. The age in terms of somites and general condition of each embryo was determined with a dissecting binocular; all embryos in which the neural tube was closed in any region and all abnormal embryos were discarded. The egg shell was marked with a pencil to indicate the position of the embryonic axis so that it could be placed parallel to the slit on the monochromator. The egg, which was tightly fixed in the watch glass by modeling clay, was placed upon a stand which had been attached to a rack and pinion. The egg was elevated until its sur-

face was beneath a quartz prism which was situated at the slit on the monochromator. This "mechanical jack" enabled the investigator to place each embryo the same distance from the source of light. The experimental embryos were irradiated for varying lengths of time with monochromatic ultraviolet light. The control embryos were treated in exactly the same manner except that a glass microscopic slide was placed in the path of the light beam so that no ultraviolet light struck the embryo. Following irradiation, the opening in the egg shell was covered with a piece of a glass cover slip and sealed with a mixture of beeswax and paraffin. The eggs were then incubated for 30 hours or longer; the glass window was always placed down in order to prevent the blastoderm from adhering to the cover slip.

After incubation, the blastoderm was removed from the yolk by cutting around the periphery of it with iridectomy scissors and lifting it off with a metal spatula. The blastoderm was washed in saline and fixed with picro-sulfuric acid. Observations were made upon embryos in alcohol, from whole mounts, and from sectioned material. The whole mounts were stained with borax carmine and the sectioned material was stained with Delafield's hematoxylin or borax carmine.

In the majority of the experiments in this investigation, monochromatic radiation was obtained by means of a large crystal monochromator, described in detail by Uber and Jacobsohn (1938). The monochromator was operated in a horizontal position. Since it was necessary to obtain a vertical beam of light in order to irradiate the embryo, a small quartz prism was placed at the slit on the monochromator. The source of light for the monochromator was a vertical mercury arc which operated at 4 amperes on a 110 volt direct current.

In order to determine the incident dose in ergs/mm.^{2} on the embryos, the monochromatic source was calibrated with a surface-type vacuum thermopile. The thermopile had been calibrated previously with a standard carbon-filament lamp (C–241) obtained from the United States Bureau of Standards. The dosage in ergs/mm.^{2} emitted by the monochromatic source was determined by comparing the deflection which it produced with that produced by the standard lamp.

In the other experiments a mercury discharge tube served as a source of radiation. It was of the Hanovia Sc–2537 type operating at 120 milliamperes and 5000 volts. The transformer was a Jefferson luminous type. Since spectral studies show that such discharge tubes frequently have an additional line around 1800 Å (Landen, 1940), a water filter was placed in the path of the beam in order to absorb the radiation of the shorter wave length. A slit of approximately the same size as that of the monochromator used in this investigation was made on the bottom of the filter in order to approximate the experimental set-up with the monochromator.

Observations

Histological studies of irradiated and control embryos

Chick embryos ranging in age from the primitive streak to the 8-somite stage were irradiated with monochromatic ultraviolet light of wave lengths 2483, 2537, 2576, 2650, 2699, 2804, 2894, 2967, and 3130 Å and subsequently incubated for a period of approximately 30 hours.

The smallest doses produce no detectable changes; the first visible effects to appear as the dose is increased are on the formation of the neural tube. The neural

folds fail to close and instead form flat or half-folded neural plates. In some embryos this occurs in the anterior part of the body; however, in other cases, it is present only in the middle portion. Large doses result in destruction of cells and death of the embryo.

In the consideration of the effect of monochromatic radiation on a developmental process one of the first questions to arise is this: Is it possible to set up a quantitative standard of measurement for comparing the effectiveness of different wave lengths? Such a standard would be a morphological *endpoint*. The procedure would be to compare doses required to attain such an endpoint. If the data are significant, the results should be the same regardless of the particular endpoint chosen. In the present study two morphological endpoints are used: (1) failure of the neural tube to close for a distance of one-third its length in 50 per cent of the cases and (2) failure of the neural tube to close for a distance of one-half its length in 50 per cent of the cases. This investigation is concerned in particular with the embryos irradiated with the amounts of energy necessary to produce these two morphological endpoints. The description is made from a study of embryos in 70 per cent alcohol, sectioned embryos, and whole mounts.

The primary effect of radiation is on the neural plate. Embryos irradiated with wave lengths 2483, 2537, 2576, 2650, 2699, 2804, and 2894 A are very uniform in appearance. A broad flat plate is present in the anterior one-third to five-sixths of the embryo; the neural tube is nearly always closed in the posterior end. The anterior end of the neural plate bends around the anterior tip of the free head and extends to the ventral surface. The optic cups and infundibulum develop from the portion of the neural plate on the ventral surface of the free head. A lens forms in most cases. A typical case with a broad flat plate, optic cups, lenses, and infundibulum is shown in a section through the anterior end of an embryo irradiated with wave length 2804 A (Plate I, Figure 1). A section at a more posterior level is shown in Plate I, Figure 2; this embryo was irradiated with wave length 2537 A. Observations show that the auditory pits are normal in appearance in every case. A group of cells which is probably the neural crest often lies adjacent to the lateral edges of the neural plate. In the region of the rhombencephalon the motor roots of the spinal nerves are present. In the posterior end of the embryo a double neural tube occurs occasionally.

The broad flat plate which is present in embryos of this group is nearly uniform in thickness except in the region of the midline. In this region the plate is thinner (Plate I, Figures 1 and 2). The volume of the broad plate is much larger than that of the neural plate of a 6-somite embryo. Mitoses are abundant on the upper surface of the plate and on the inside portion of the closed region of the neural tube. In a few embryos a small group of cells is present on the surface at the lateral edges of the neural plate. They are filled with granules and irregular in shape. In these cases the lateral ectoderm is similar in appearance to these cells; otherwise, the lateral ectoderm appears normal. In some cases it continues to grow and expand so that a projecting group of cells forms on the dorsal surface of the neural plate.

Another group of embryos is characterized by the presence of a flat or folded neural plate in the middle portion of the body. A complete neural tube forms in the anterior part of the embryo, but it is abnormal in shape and smaller than a

PLATE I

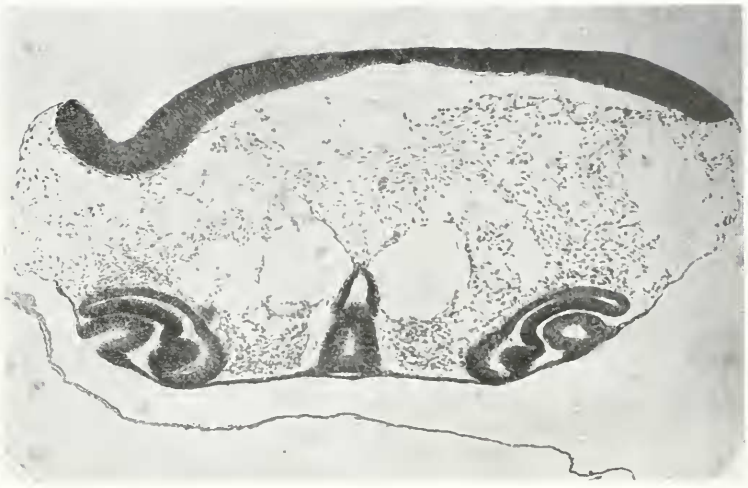

FIGURE 1. Anterior end of an embryo irradiated with approximately 71 ergs/mm.^{2} at wave length 2804 Å and subsequently incubated for 30 hours, showing the broad flat neural plate, optic cups, lens, infundibulum, gut, dorsal aortae, small notochord, and disorganized mesenchyme beneath the neural plate. × 107.

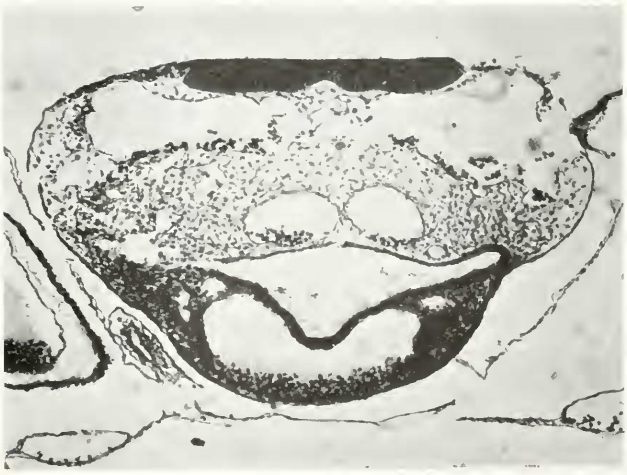

FIGURE 2. At the level of the heart of an embryo irradiated with approximately 95 ergs/mm.^{2} at wave length 2537 Å and subsequently incubated for 30 hours, showing the flat neural plate which possesses a well-defined floor plate, small notochord, dorsal and ventral aortae, heart, and gut. × 107.

normal tube. The typical thin roof plate of the myelencephalon fails to develop. The auditory pits and optic cups are normal.

The cellular appearance of the notochord in the irradiated embryos is the same as that of a normal embryo. Measurements of the cross-section area of the notochord show that it varies greatly at different anterior-posterior levels. The notochord is invariably separated from the neural plate; in the region of the neural tube the notochord lies in contact with the floor plate. In Plate I, Figures 1 and 2 illustrate the small size and relative position of the notochord. As far as can be detected, the somites are normal. In many cases, the mesenchyme beneath the neural plate is abnormally vesiculated in places and considerably disorganized (Plate I, Figure 1). Stained sections show that the mesenchyme beneath the neural plate has been injured; this is suggested by the dark appearance of its cells. The vascular system is well developed; however, the size of the vascular bed is smaller than that of control embryos.

The embryos which were irradiated with wave length 2967 Å are different in appearance from those described previously. The explanation for this is that extremely large doses had to be used in order to produce a detectable effect. In most cases the neural tube forms only in the most anterior part of the embryo. It is very small and abnormally shaped, being extremely flattened dorsoventrally. Occasionally the tube fails to develop and a neural plate is present in the anterior region. In the posterior three-fourths to four-fifths of the body the neural plate is either a disorganized mass of cells or completely absent. The superficial ectoderm appears normal and forms a continuous layer of flat epithelial cells dorsal to the neural plate.

In the embryos irradiated with wave length 2967 Å, the cellular structure of the notochord is normal in appearance. However, the notochord is not in contact with either the neural tube or plate in most regions. The somites are either highly disorganized or absent. The vascular system is poorly developed.

Observations of 111 control embryos which were made from embryos in 70 per cent alcohol, whole mounts, and sections show that they are normal in 100 per cent of the cases. It will be remembered that all embryos were examined immediately before irradiation and the abnormal ones discarded. This explains the fact that all control embryos are normal.

From this description, it is evident that all wave lengths except 2967 Å produce a uniform effect on neural tube formation; consequently, quantitative studies of the relative efficiency of different wave lengths in preventing closure of the neural tube can be made.

The effect of radiation on mitosis and volume of the central nervous system

The purpose of this study is to determine if radiation has a detectable influence on mitosis and volume changes with the low doses used. Mitotic counts and measurements of the area of cross sections of the central nervous system were made on the embryos which were described histologically in the previous section. An analysis of the effect of radiation on mitosis was made by counting the number of mitoses in three sections of the neural plate or tube at each of three different levels. Volume was measured indirectly by determining the cross-section area of one of

TABLE I

Mitotic counts and cross-section measurements of irradiated and control embryos

Group of embryos	Number of mitoses per section per embryo	Cross-section area per embryo	Mitoses per cross-section area unit
Level *a*			
Embryos irradiated at wave lengths from 2483 to 2804 Å (10)*	45.90±14.7	1189.2±278	.038
Embryos irradiated at wave lengths 2894 and 2967 Å (4)	20.75±9.0	719.5±95	.029
Control embryos (5)	44.80±15.0	1504.6±409	.030
Level *b*			
Embryos irradiated at wave lengths from 2483 to 2804 Å (10)	7.80±2.6	251.3±34	.031
Embryos irradiated at wave lengths 2894 and 2967 Å (4)	1.75±2.1	93.75±47	.018
Control embryos (5)	12.60±4.7	379±115	.033
Level *c*			
Embryos irradiated at wave lengths from 2483 to 2804 Å (10)	6.90±2.1	191.5±51	.036
Embryos irradiated at wave lengths 2894 and 2967 Å (4)	4.75±5.6	99.5±85	.048
Control embryos (5)	10.00±2.0	284.60±46	.035

Level *a* consists of three sections adjacent to the anterior end of the notochord; level *b* is represented by the middle section of the central nervous system and this level is determined by counting the total number of sections of the central nervous system; and level *c* is ten sections posterior to the most posterior section in which the neural tube has failed to close in the irradiated embryos and a comparable section in control embryos. In the irradiated embryos in which the counts were made, level *c* is always in the posterior quarter of the nervous system. In determining the number of mitoses per section per embryo, counts were made on three sections at each level of each embryo. The number of mitoses per section at each level was obtained by averaging these three figures; the number of mitoses per section per embryo was determined by averaging the number of mitoses per section for the group of embryos. Volume was measured by determining the cross-section area of one of the sections at each level in which the mitoses were counted; cross-section area per embryo was obtained by averaging the cross-section area for the group of embryos. The mean deviation has been calculated for the average values of the number of mitoses and cross-section area.

* The figures in parenthesis represent the total number of embryos studied in each group.

the sections at each level in which the mitoses were counted. This was accomplished by tracing the outline of the neural plate or tube on millimeter paper by means of a camera lucida and then counting the number of square millimeters

TABLE II

The effect of an increased incubation period on closure of the neural tube

Egg number	Irradiated with 143.6 ergs/mm.²				Irradiated with 170.0 ergs/mm.²			
	30-hour incubation		54-hour incubation		30-hour incubation		54-hour incubation	
	Age in somites at time of irradiation	Distance open	Age in somites at time of irradiation	Distance open	Age in somites at time of irradiation	Distance open	Age in somites at time of irradiation	Distance open
1	6	1/3	6	1/3	6	1/2	6	0
2	6	1/1	4	1/3	3	1/2	5	1/5
3	6	3/1	6	0	2	1/2	6	0
4	6	0	5	0	6	1/5	6	1/4
5	6	open	6	0	6	1/2	6	0
6	6	1/3	1	0	0	0	6	0
7	6	1/3	7	0	7	1/4	3	1/4
8	5	0	4	0	7	3/4	4	1/3
9	6	open	4	0	6	2/3	7	1/5
10	3	open	6	0	6	1/3	6	open
11	2	3/1	6	0	8	1/1	8	1/4
12	3	1/3	6	0	2	0	3	0
13	0*	0	6	0	3	3/1	5	1/2
14	3	0	4	1/3	2	1/2	7	0
15	6	1/2	6	1/3	2	3/1	6	1/2
16	0	0	6	0	3	1/2	7	0
17	6	1/3	5	1/1	7	1/3	6	0
18			6	0			7	0
19			6	0			7	1/3
20							6	1/3
21							6	0
22							8	0
23							4	0
24							6	1/3
25							7	1/6
% open 1/3 way or more		47		21		70		26

* Embryos designated as having no somites were in the primitive streak stage.

within the outline. No attempt was made to transfer the values obtained into absolute ones, since this investigation is concerned only with relative data.

The results are shown in Table I. From the data presented, it is concluded that the results with wave lengths 2483 to 2804 Å are not decisive enough to establish an influence of radiation on mitosis and volume. However, wave lengths 2894 and 2967 Å are very effective in decreasing mitosis and volume.

The effect of an increased incubation period on closure of the neural tube

The purpose of this experiment is to determine whether or not the neural folds which have failed to close in irradiated embryos will close if the incubation period is increased. Embryos ranging in age from the primitive streak to the 8-somite stage were irradiated with the mercury discharge tube with doses of 143.6 and 170.0 ergs/mm.^{2}. The embryos were then incubated for periods of 30 and 54 hours. After removal and fixation of the embryos, observations were made with a dissecting binocular. The results are shown in Table II. Columns 3, 5, 7, and 9 which are designated as "Distance open" refer to the distance for which the neural tube has failed to close. The per cent of embryos in which the neural tube is open for a distance of one-third its length or more decreases with an increase in the period of incubation. For the 143.6 ergs/mm.^{2} group the per cent decreases from 47 to 21; in the 170.0 ergs/mm.^{2} group the per cent decreases from 70 to 26. This experiment shows that an increased incubation period results in partial closure of the region of the central nervous system which had failed to close after 30 hours incubation.

Method of calculation of the incident energy on the embryos

In the histological study of the irradiated embryos described in the first section of the observations, all wave lengths except 2967 Å are found to produce the same

TABLE III

Absolute intensity of monochromatic radiation

Wave length	Cm. deflection	Ergs/mm.^{2}/sec. producing 1 cm. deflection	Ergs/mm.^{2}/sec.
2483	1.5	.726	1.089
2537	5.2	.726	3.775
2576	.6	.726	.436
2650	3.8	.726	2.759
2699	.9	.726	.653
2804	2.1	.726	1.525
2894	1.1	.726	1.799
2967	2.9	.726	2.105
3130	12.5	.726	9.075

qualitative effects. Since the effect of radiation is to inhibit the folding process, a quantitative comparison of the relative photochemical efficiency of different wave lengths can be made. The amount of incident energy in ergs/mm.^{2} required to inhibit folding was determined for the wave lengths used in this investigation. To facilitate a comparison of the relative efficiency of these wave lengths, two morphological endpoints, namely, the inhibition of closure of the neural tube for a distance of one-third and for a distance of one-half its length in 50 per cent of the embryos were chosen. *Attention is called to the fact that these morphological endpoints have dimensions of energy and for this reason are an indirect measure of absorption.* In order to determine the incident energy on the eggs, the intensity of the

radiation at the egg surface for each wave length was measured by means of a surface-type vacuum thermopile. The thermopile measurements are shown in Table III, where "cm. deflection" refers to the number of centimeters the galvanometer needle was deflected when the intensity of each wave length was measured. By multiplying this value by .726 ergs/mm.²/sec., which is the amount of energy producing a deflection of 1 cm., the number of ergs/mm.²/sec. emitted by each line was obtained.

The total numbers of embryos irradiated at wave lengths 2483, 2537, 2576, 2650, 2699, 2804, 2894, 2967, and 3130 Å are 75, 93, 45, 100, 68, 79, 42, 54, and 16, re-

TABLE IV

Results with experimental doses stronger and weaker than the endpoint dose

Wave length	Incident dose on egg in ergs/mm.²	Open 1/3 way or more	Open less than 1/3 way or closed	Per cent open 1/3 way or more	Open 1/2 way or more	Open less than 1/2 way or closed	Per cent open 1/2 way or more
2483	196.06	16	25	39	13	28	32
	261.36	10	9	53	6	13	32
2537	113.25	4	14	22			
	169.89	19	9	68	11	17	39
	226.50				9	6	60
2576	130.80	10	12	45	5	17	23
	156.96	13	5	72	11	7	61
2650	207.00	14	16	47	6	24	20
	248.40	14	9	60	12	11	52
2699	195.90	4	6	40			
	215.49	2	2	50			
	235.08	11	10	52	10	11	48
	274.26				4	1	80
2804	137.25	17	17	50	15	19	44
	183.00				4	1	80
2894	239.70	6	10	38	5	11	31
	287.64	12	6	67	8	10	44
2967	631.50	0	13	?	0	13	?
	757.80	0	17	?	0	17	?
3130	5445.00	0	7	?	0	7	?
	9256.50	0	1	?	0	1	?

The figures in columns 3, 4, 6, and 7 represent the number of embryos irradiated; the figures in columns 5 and 8 refer to the per cent of embryos irradiated. "Open one-third way" or "open one-half way" means that the neural tube is open at least one-third or one-half its length; "open" refers to cases in which the neural tube is open less than one-third or one-half way; and "closed" indicates that the neural tube is closed.

spectively. The results obtained with incident doses on the egg stronger or weaker than the doses required to inhibit closure of the neural tube for distances of one-third and one-half its length in 50 per cent of the cases are summarized in Table IV.

In order to determine the amount of energy incident on the embryo, it is necessary to measure the amount of light transmitted by the vitelline membrane. The ultraviolet transmission of the vitelline membrane was measured by Uber, Hayashi, and Ellis (1941). These measurements were made with a Spekker photometer and a Hilger medium quartz spectrograph. Three vitelline membranes were studied and the results are shown in numerical form in Table V for each wave

TABLE V

Ultraviolet transmission of three vitelline membranes

(Hilger spectrograph data)

Wave length	Per cent transmission			
	Membrane 1	Membrane 2	Membrane 3	Average
2483	6.35	4.65	3.05	4.68
2537	8.00	6.20	4.20	6.13
2576	9.90	7.00	5.20	7.37
2650	10.00	7.80	6.80	8.20
2699	10.00	8.00	7.45	8.48
2804	11.00	8.10	8.00	9.03
2894	16.30	13.65	11.00	13.65
2967	21.80	20.70	15.85	19.45
3130	26.10	30.10	30.10	28.76

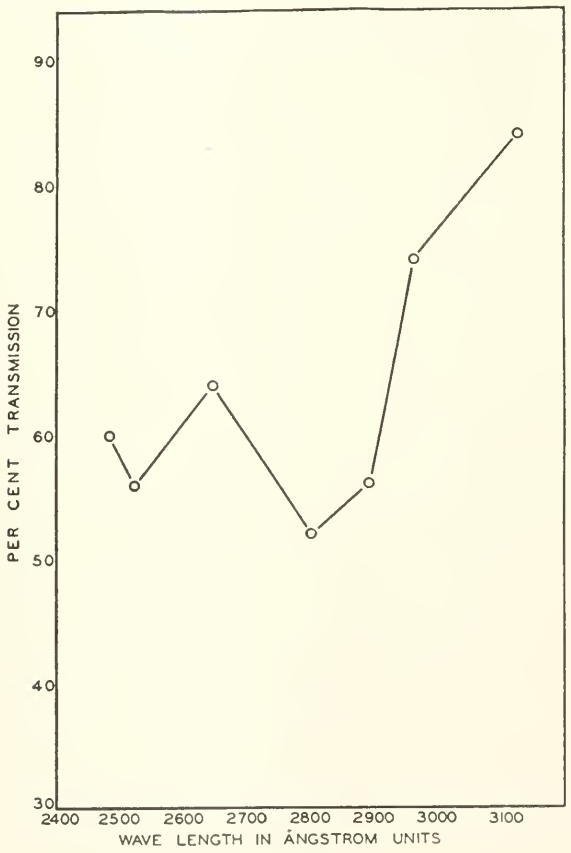

FIGURE 1. Ultraviolet transmission by vitelline membrane (T. Hayashi, unpublished).

length used in this study. In addition to the spectrograph data, Mr. Teru Hayashi (unpublished) measured the ultraviolet transmission of the vitelline membrane with a photocell and a quartz microscope. His data are shown in Figure 1. The amount of energy incident on the embryo was then calculated for these two sets of data on transmission by the vitelline membrane by multiplying the per cent transmission by the vitelline membrane by the incident energy on the egg.

Relative photochemical efficiency curves

The results of the calculations of the incident energy on the embryos are presented in Tables VI and VII in which correction for absorption by the vitelline membrane is made with the Hilger spectrograph; in Tables VIII and IX, the

TABLE VI

Incident energy on embryos for inhibition of closure of ⅓ the length of the neural tube
(Hilger spectrograph data)

Wave length	Incident dose on egg in ergs/mm.²	Per cent transmission by vitelline membrane	Per cent open ⅓ way	Incident dose on embryo in ergs/mm.²	Reciprocal of incident dose on embryo	Calculated endpoint dose	Reciprocal of calculated endpoint dose
2483	196.06	4.68	39	9.18	.109	11.58	.086
	261.36		53	12.23	.082		
2537	113.25	6.13	22	6.84	.146	9.01	.110
	169.89		68	10.41	.096		
2576	130.80	7.37	45	9.64	.104	10.00	.100
	156.96		72	11.57	.086		
2650	207.00	8.20	47	16.97	.059	17.75	.056
	248.40		60	20.37	.049		
2699	195.90	8.48	40	16.61			
	215.49		50	18.27	.055	18.27	.055
	235.08		52	19.93			
2804	137.25	9.03	50	12.39	.080	12.39	.080
2894	239.70	13.65	38	32.62	.031	35.37	.028
	287.64		67	39.26	.025		
2967	631.50	19.45	?	122.83	.0081	?	?
	757.80		?	147.39	.0068	?	?
3130	5445.00	28.76	?	1565.98	.0006	?	?
	9256.50		?	2662.17	.0004	?	?

photocell and quartz microscope transmission measurements are used for this correction. At most wave lengths the doses recorded in the tables are just stronger or weaker than the endpoint doses, namely, the amounts of energy necessary to prevent closure of the neural tube for distances of one-third and one-half its length in 50 per cent of the embryos. At wave length 2804 Å, a dose was used which prevented neural tube formation in exactly 50 per cent of the cases when one-third the length of the tube was used as an endpoint. When the doses used did not produce the endpoint, the endpoint dose was determined by interpolation. The validity of this interpolation is based upon the assumption that the effect produced is directly proportional to dose. This assumption was used during the course of this investigation to predict the dose which would produce the endpoint. These predictions were fairly accurate, particularly in view of the small number of em-

TABLE VII

Incident energy on embryos for inhibition of closure of ½ the length of the neural tube
(Hilger spectrograph data)

Wave length	Incident dose on egg in ergs/mm.²	Per cent transmission by vitelline membrane	Per cent open ½ way	Incident dose on embryo in ergs/mm.²	Reciprocal of incident dose on embryo	Calculated endpoint dose	Reciprocal of calculated endpoint dose
2483	196.06	4.68	32	9.18	.109	?	?
	261.36		32	12.23	.082		
2537	169.89	6.13	39	10.41	.096	12.12	.082
	226.50		60	13.88	.072		
2576	130.80	7.37	23	9.64	.104	11.01	.090
	156.96		61	11.57	.086		
2650	207.00	8.20	20	16.97	.059	20.16	.050
	248.40		52	20.37	.049		
2699	235.08	8.48	48	19.93	.050	20.20	.050
	274.26		80	23.25	.043		
2804	137.25	9.03	44	12.39	.081	13.08	.076
	183.00		80	16.52	.061		
2894	239.70	13.65	31	32.62	.031	42.32	.023
	287.64		44	39.26	.025		
2967	631.50	19.45	?	122.83	.0081	?	?
	757.80		?	147.39	.0068		
3130	5445.00	28.76	?	1565.98	.0006	?	?
	9256.50		?	2662.17	.0004		

TABLE VIII

Incident energy on embryos for inhibition of closure of ⅓ the length of the neural tube
(Quartz microscope and photocell data)

Wave length	Incident dose on egg in ergs/mm.²	Per cent transmission by vitelline membrane	Per cent open ⅓ way	Incident energy on embryo in ergs/mm.²	Reciprocal of incident dose on embryo	Calculated endpoint dose	Reciprocal of calculated endpoint dose
2483	196.06	60	39	117.63	.0085	148.42	.0067
	261.36		53	156.82	.0064		
2537	113.25	56	22	63.42	.0158	82.75	.0121
	169.89		68	95.14	.0105		
2576	130.80	58.5	45	76.52	.0131	79.35	.0126
	156.96		72	91.82	.0109		
2650	207.00	64	47	132.48	.0075	137.78	.0073
	248.40		60	158.98	.0063		
2699	195.90	59	40	115.58	.0087		
	215.49		50	127.14	.0080	124.14	.0080
	235.08		52	138.69	.0073		
2804	137.25	52	50	71.37	.0140	71.37	.0140
2894	239.70	56	38	134.20	.0074	145.32	.0069
	287.64		67	161.08	.0062		
2967	631.50	74	?	467.31	.0021	?	?
	757.80			560.77	.0018		
3130	5445.00	84	?	4573.80	.00022	?	?
	9256.50		?	7775.46	.00013		

bryos obtained at stronger and weaker doses which served as a basis for the predictions. In most cases three or four experiments were run and comparable results were obtained in each experiment. Furthermore, if the experimental data which most closely correspond to the endpoint are used instead of the interpolated data, the maxima and minima of the curves are not significantly changed.

Although the embryos at the time of irradiation varied in age from the primitive streak to the eight somite stage, the results were not altered. Davis (1942) recorded the age of each embryo at the time of irradiation and the results show that the effect of radiation is independent of age for the small age range used in this

TABLE IX

Incident energy on embryos for inhibition of closure of ½ the length of the neural tube
(Quartz microscope and photocell data)

Wave length	Incident dose on egg in ergs/mm.²	Per cent transmission by vitelline membrane	Per cent open ½ way	Incident energy on embryo in ergs/mm.²	Reciprocal of incident dose on embryo	Calculated endpoint dose	Reciprocal of calculated endpoint dose
2483	196.06	60	32	117.63	.0085	?	?
	261.36		32	156.82	.0064		
2537	169.89	56	39	95.14	.0105	111.74	.0089
	226.50		60	126.84	.0079		
2576	130.80	58.5	23	76.52	.0131	87.39	.0114
	156.96		61	91.82	.0109		
2650	207.00	64	20	132.48	.0075	157.32	.0064
	248.40		52	158.98	.0063		
2699	235.08	59	48	138.69	.0073	153.22	.0065
	274.26		80	161.81	.0062		
2804	137.25	52	44	71.37	.0140	75.34	.0133
	183.00		80	95.16	.0105		
2894	239.70	56	31	134.2	.0074	172.71	.0058
	287.64		44	161.08	.0062		
2967	631.50	74	?	467.31	.0021	?	?
	757.80			560.77	.0018	?	?
3130	5445.00	84	?	4573.80	.00023	?	?
	9256.50		?	7775.46	.00013		

group of experiments. Furthermore, it was possible to predict the experimental results that would be obtained with slightly larger or smaller doses regardless of the age of the embryos used.

Interpolations cannot be made at wave lengths 2967 and 3130 Å. A large percentage of the tubes are open in the embryos which were irradiated with wave length 2967 Å; however, none are open as much as one-third the length of the neural tube. Embryos irradiated with 495.20 ergs/mm.² or less show a slight injury, while the stronger doses used produce considerable injury. In view of this and since 757.80 ergs/mm.² result in failure of the neural tube to close in 35 per cent of the cases, a dose of approximately 757.80 ergs/mm.² is chosen as an endpoint dose. Wave length 3130 Å is found to be ineffective since a dose of 5445.00 ergs/mm.² fails to produce a detectable change in the embryos.

The reciprocal of the calculated endpoint dose in ergs/mm.² is plotted against wave length for each of the four sets of data and the photochemical efficiency curves are presented in Figures 2, 3, 4, and 5. All four curves show two well-defined ab-

sorption maxima at wave lengths 2576 and 2804 Å and a minimum at wave lengths 2650–2700 Å. The validity of using a morphological endpoint as a measure of the effect produced is shown by the fact that the curves for the two different morphological endpoints are almost identical in shape. Furthermore, the curves are very similar in shape regardless of the data used in correction for absorption by the vitelline membrane. This shows that the differences in effectiveness of different wave lengths as shown in the photochemical efficiency curves are truly significant.

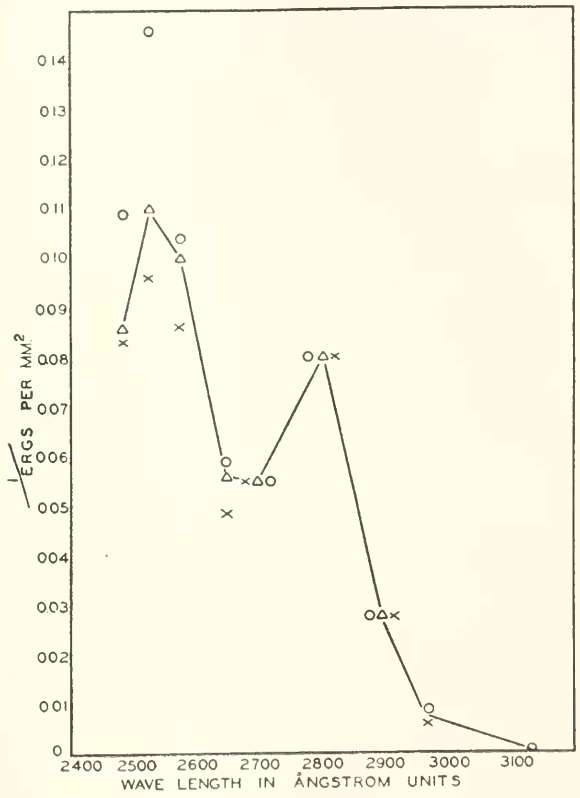

FIGURE 2. Photochemical efficiency curve. Inhibition of closure of the neural tube for a distance of at least one-third its length in 50 per cent of the embryos. Correction for extinction by vitelline membrane based upon transmission measurements with Hilger spectrograph. (○) and (×) are symbols for experimentally determined doses stronger and weaker respectively than the calculated end-point doses (△).

DISCUSSION

Histological studies were made in order to determine whether a comparable unit of ultraviolet effect on the folding process was obtained at all effective wave lengths. Observations of the embryos irradiated with the amounts of energy necessary to produce the two morphological endpoints, namely, failure of the neural tube to close for distances of one-third and one-half its length in 50 per cent of the cases,

show that the most apparent effect of ultraviolet light is on the neural plate. Instead of a neural tube forming, a broad flat plate develops. In a few cases the lateral ectoderm appears to be injured as suggested by the dark granular appearance of its cells. The groups of cells observed on the surface of the neural plate are probably derived from the lateral ectoderm since the two are very similar in appearance. If this is true, then the isolation of these groups of cells likewise indicates that the lateral ectoderm is injured. However, the magnitude of this effect

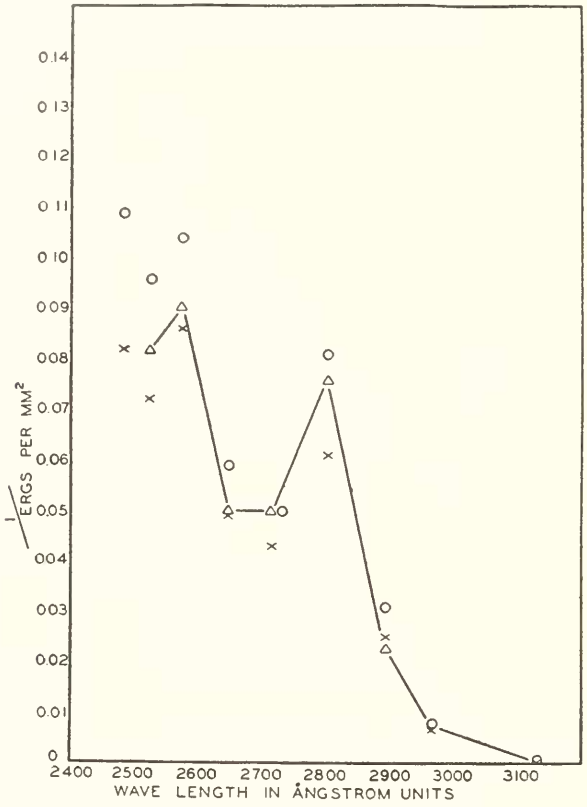

FIGURE 3. Photochemical efficiency curve. Inhibition of closure of the neural tube for a distance of at least one-half its length in 50 per cent of the embryos. Correction for extinction by vitelline membrane based upon transmission measurements with Hilger spectrograph. (○) and (×) are symbols for experimentally determined doses stronger and weaker respectively than the calculated end-point doses (△).

is small; otherwise, the lens and otic vesicle would not develop in an apparently normal manner. Consequently, the effect of radiation on the ectoderm is not nearly as great on the lateral ectoderm as it is on the neural plate.

Only mesodermal derivatives which lie directly beneath the ectoderm are injured. Although no detectable change in the cellular appearance of the notochord was observed, the cross-section measurements indicate that the notochord is affected. The mesenchyme beneath the ectoderm is also injured. This interpreta-

tion is made from the dark and disorganized appearance of the mesenchyme cells. The somites are normal in appearance. It should be emphasized that the effects of radiation on the ectoderm and mesoderm discussed previously are uniform for all wave lengths except 2967 Å.

As previously stated, the most apparent effect of ultraviolet light is to prevent closure of the neural tube. Furthermore, failure of the neural plate to form a

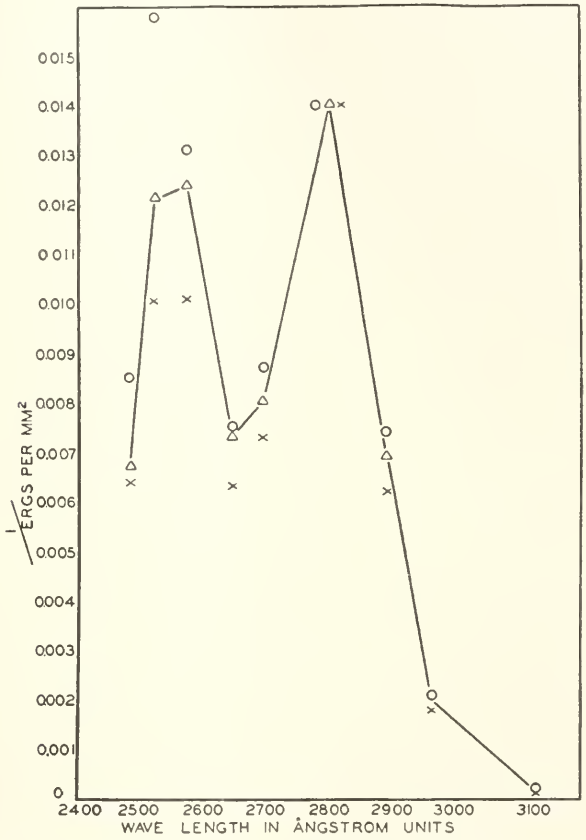

FIGURE 4. Photochemical efficiency curve. Inhibition of closure of the neural tube for a distance of at least one-third its length in 50 per cent of the embryos. Correction for extinction by vitelline membrane based upon transmission measurements with the photocell and quartz microscope. (○) and (×) are symbols for experimentally determined doses stronger and weaker respectively than the calculated end-point doses (△).

neural tube seems to be restricted to an effect upon the folding process because radiation does not appear to produce other effects on the neural plate cells. The results presented in Table II show that prolonged incubation leads to closure of the neural tube in regions which were open after 30 hours incubation. This probably can best be interpreted as indicating that material and not the capacity to produce or utilize material is altered. Microscopic observations fail to reveal any abnormal changes in the cells of the neural tube. The volume of the neural plate or

tube has increased considerably. In certain regions, the motor roots of the spinal nerves grow out of the neural plate which shows that histological differentiation continues even though folding of the plate as a whole has been inhibited.

Mitoses are very abundant and normal in position. The quantitative determinations presented in Table I suggest that mitosis is slightly affected by radiation at wave lengths from 2483 to 2804 Å. However, enough data have not been

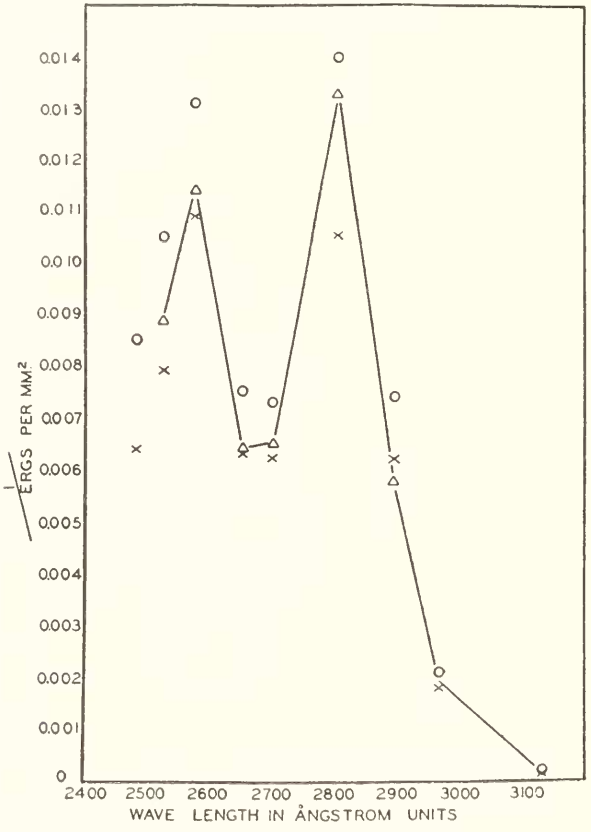

FIGURE 5. Photochemical efficiency curve. Inhibition of closure of the neural tube for a distance of at least one-half its length in 50 per cent of the embryos. Correction for extinction by vitelline membrane based upon transmission measurements with the photocell and quartz microscope. (○) and (×) are symbols for experimentally determined doses stronger and weaker respectively than the calculated end-point doses (△).

analyzed to settle this question. In view of the evidence presented by others on the effect of radiation on avian material, it seems unlikely that radiation has an effect at these small doses. Mayer and Schreiber (1934) found that doses of radiation several times as large as those used in this investigation were required to inhibit cell division of chick fibroblasts and chondroblasts in tissue culture. Even if mitosis is affected, the energy involved in producing this effect is evidently not involved in preventing folding. This reasoning is based upon the observation that

doses at wave lengths 2894 and 2967 Å which affect mitosis do not influence folding. The same inverse relation is found in the case of the cross-section measurements. Consequently, the data which have been analyzed indicate that a decrease in mitosis and volume is not causally related to inhibition of the folding process. This suggests that the incident radiation which is required to prevent folding is not involved in nuclear or volume changes and, consequently, is not absorbed by the cellular material engaged in these changes. From the discussion of the histological observations, mitotic counts, and cross-section measurements, it is evident that a comparable unit of ultraviolet effect on inhibition of the folding process is obtained at different effective wave lengths. In view of this, it is possible to obtain a photochemical efficiency curve for this process.

In order to determine accurately the photochemical efficiency of different wave lengths, corrections have been made for the energy absorbed by the material which screens the embryo. The two possible sources of error are the albumin and the vitelline membrane. The albumin present above the embryo after a 24 hour incubation period is negligible. It is unlikely that any albumin present is absorbing incident radiation since wave length 2804 Å which is most effective in inhibiting folding is most strongly absorbed by albumin.

On the other hand, the vitelline membrane absorbs a large per cent of the incident light. As can be seen by an examination of the data for the transmission measurements, different results were obtained by the two methods. With the spectrograph method only the amount of light transmitted by the vitelline membrane is measured and the values obtained by calculation of the incident energy on the embryo are minimum. The photocell and quartz microscope measure not only transmitted but some scattered radiation. Since the vitelline membrane lies in contact with the surface of the embryo, most of the scattered radiation is likely absorbed. In view of this the photocell measurements give a better insight into the actual amount of energy incident on the embryo. It should be pointed out, however, that the exact amount of scattered radiation measured with the photocell and quartz microscope is dependent upon certain experimental conditions such as the distance of the object from the objective and the diameter of the opening in the iris diaphragm. For this reason the photocell data can be relied on to give only an approximate value for the incident energy on the embryo.

Since the photocell and quartz microscope transmission measurements give a better measure of the incident energy on the embryo, the efficiency curves obtained by correction with the photocell data will be considered in the comparison with absorption spectra. Although the curves drawn between the points determined by interpolation are based upon the assumption that the morphological effect is proportional to dose, this does not influence the magnitude of the absorption maxima and minima since the two experimental points are close together at most wave lengths.

As was pointed out in the first part of the discussion, the significance of the relative photochemical efficiency curves lies in the fact that they can be used to determine the chemical nature of the irradiated material. This is possible since photochemical efficiency curves are an indirect measure of the amount of energy absorbed when certain fundamental assumptions are fulfilled. These assumptions are: (1) radiation must be transmitted by the absorbing system; (2) the quantum yield for the substance absorbing the energy must be the same for all effective wave

lengths; and (3) only the radiation which is absorbed and involved in producing the photochemical effect is measured. The validity of this technique was proved both mathematically and experimentally by Warburg (1927) and (1930). Since then, several investigators, namely, Gates (1930), Oster (1935), Giese (1938), and Landen and Uber (1939), have used this method.

An examination of the validity of the data in the present investigation with reference to the three assumptions shows that the first assumption is supported by two types of evidence. From cross-section measurements of the notochord, it appears that radiation affects its shape. Since the notochord lies directly beneath the neural plate, it is suggested that radiation is transmitted by the neural plate. This type of reasoning, however, is subject to the criticism that the notochord might be affected indirectly through the action of radiation on the neural plate or other structures. More conclusive evidence is presented by the dark and disorganized appearance of the mesenchyme cells beneath the neural plate.

From the experiments in this study data concerning the constancy of quantum yield with wave length are not available. In order to ascertain the quantum yield of the material engaged in folding it would be necessary to know the particular compound involved. In this investigation no attempt is made to determine the exact chemical compound but only to ascertain to what general group of compounds the material belongs. It is interesting to note that Harris, Bunker, and Mosher (1938) found the quantum yield for ergosterol to be constant for wave lengths 2537, 2652, 2894, 2967, and 3025 Å. Bunker, Harris, and Mosher (1940) repeated this experiment for 7-dehydrocholesterol and found a uniform quantum efficiency for all wave lengths except 2967 Å which was slightly more efficient. In a study of proteins, Landen (1940) and Hollaender and Duggar (1936) found the quantum yield to be constant for wave lengths between 2400 and 3130 Å.

Evidence for fulfillment of the third assumption will now be considered. As shown in unpublished work by the author, inhibition of the folding process is almost exclusively the result of an effect upon the neural plate cells. Furthermore, a very small amount of neural plate material seems to be affected by the energy involved in inhibiting folding since effects on mitosis and volume of the neural plate do not appear to influence the folding process. Consequently, it can be concluded that if other molecules are absorbing some of the incident energy required to prevent closure of the neural tube, the amount absorbed must be very small. The incident dose at wave length 2804 Å required to produce the morphological effect on the neural tube is approximately 71 ergs/mm.^{2}. When the incident energy is expressed in quanta, a value of 1.1×10^{13} quanta/mm.^{2} is obtained. If this figure is divided by the number of cells in a square millimeter of the neural plate which is of the order of 10^3 to 10^4, a value of 1.1×10^9 to 10^{10} quanta is obtained for the incident energy on one cell. This is a smaller dose than that required to produce just a perceptible retardation of cleavage in one sea urchin egg. Giese (1938) found that an incident energy of 623.36 ergs/mm.^{2} or 3.74×10^{11} quanta per egg at wave length 2537 Å was the smallest dose which would retard cleavage and that wave length 2804 Å was only twice as effective as wave length 2537 Å. In view of this, it can be safely concluded that no more than a very small amount of energy is absorbed by molecules "screening" the inactivated material.

Since this technique is valid within the limitations of the data, it is possible to compare the relative photochemical efficiency curves with the absorption spectra of

chemical compounds. A very extensive series of absorption spectra of the most important biological substances was compiled from the data of several hundred investigators and published by Ellinger (1937; 1938). The major groups of compounds included were fats, carbohydrates, proteins, sterols, phosphatides, carbonic acid and its derivatives, alkaloids, and glycosides. The photochemical efficiency curves obtained in this study possess absorption maxima at wave lengths 2576 and

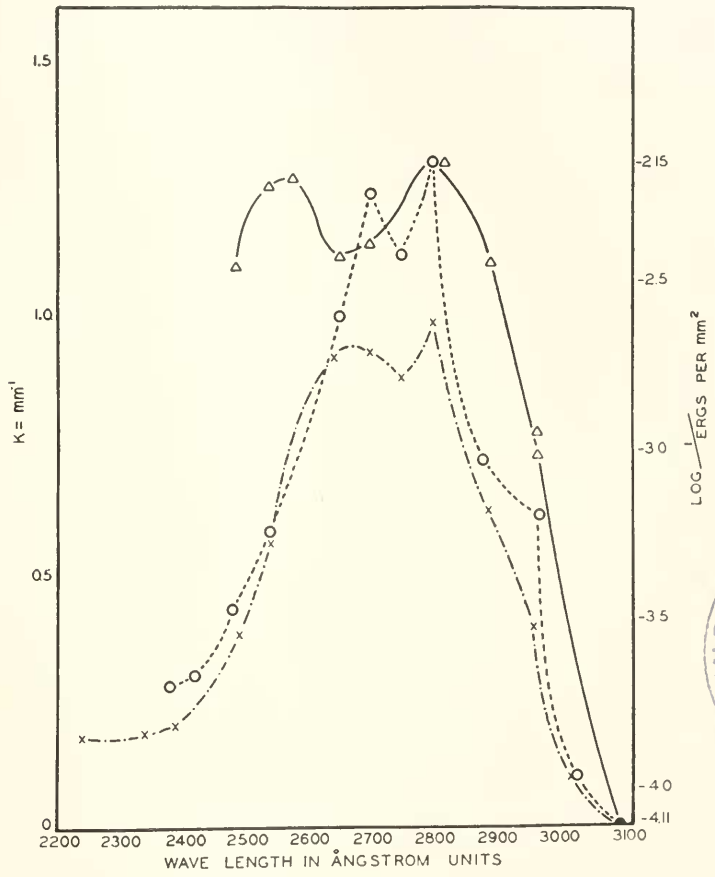

FIGURE 6. The photochemical efficiency curve for inhibition of the folding process in neural tube formation (—△—) compared with the absorption spectra of lumisterol (—·—×—·—) and 7-dehydrocholesterol (---○---).

2804 Å and a minimum at wave lengths 2650–2700 Å. Comparison of these curves with the absorption spectra of biological compounds shows that the efficiency curves resemble very closely the absorption spectra of certain sterols. The sterol curves which are very similar to the efficiency curves are for three vitamin D precursors, namely, 7-dehydrocholesterol, ergosterol, and lumisterol. In Figure 6, the photochemical efficiency curve for inhibition of closure of the neural tube for a distance of at least one-third its length when correction is made for absorption by the vitel-

line membrane based upon the quartz microscope and photocell data is compared with the absorption spectra of two of these vitamin D precursors.

No attempt is made to compare the efficiency curve with a particular sterol curve since absorption curves of very closely related compounds are known to vary, particularly in the position of the maxima. Von Dimroth (1939) found that the absorption spectra of sterols are dependent upon the number and location of double bonds. The position of the absorption maxima is dependent also upon whether the double bonds are located in one or in two rings. Furthermore, the solvent and pH of the mixture play a role in the position of the absorption bands. It is also possible that the chemical material which has been irradiated is an unidentified sterol or group of sterols. Consequently, an exact match of the curves cannot be expected.

Attention is called to the fact that the photochemical efficiency curves do not resemble absorption spectra of single proteins. Most protein curves have only one maximum which is at 2800 Å and a minimum around 2500 Å which is the position of the secondary maximum in the efficiency curve presented in Figure 6. It should be mentioned, however, that a mixture of nuclear and cytoplasmic proteins might give an absorption curve similar to the photochemical efficiency curves. This is unlikely because it would involve the photochemical inactivation of two different compounds simultaneously. Furthermore, nucleoproteins which are involved in the mitotic mechanism do not seem to be involved in folding because the wave lengths which are most effective in inhibiting mitosis are least effective in inhibiting folding.

The part that sterols play in early development is discussed by Needham (1942). He calls attention to their possible role in development as "neurogens," i.e. substances which stimulate gastrula ectoderm to neural differentiation. From the present investigation it is evident that sterols are very probably involved at a slightly later stage in development, namely, in neural tube formation. Although the technique used in this study does not allow a final or conclusive identification of a specific compound to be made, it does give (1) evidence that a special compound is involved in folding that does not seem to be engaged in other morphogenetic processes at this time in development and (2) a very strong indication as to the chemical nature of this material.

Summary

1. Chick embryos ranging in age from the primitive streak to the 8-somite stage were irradiated with monochromatic ultraviolet radiation of wave lengths 2483 to 3130 Å and subsequently incubated for 30 hours.

2. Histological studies show that radiation inhibits the folding process in neural tube formation, while cell division and volume changes continue. This effect on the neural plate is uniform for all wave lengths except 2967 Å; nevertheless, wave length 2967 Å inhibits folding.

3. After correction for absorption by the vitelline membrane, the incident energy on the embryos required to inhibit the folding process was calculated. Folding is affected by all wave lengths except 3130 Å and photochemical efficiency curves for the folding process are presented.

4. In order to obtain information concerning the chemical nature of the mate-

rial involved in folding, the photochemical efficiency curves which are an indirect measure of absorption were compared to the absorption spectra of biological compounds. The validity of this technique is based upon three fundamental assumptions which are satisfied within the limitations of the available data.

5. The photochemical efficiency curves are very similar to the absorption spectra of sterols, particularly vitamin D precursors. The small doses used in the inhibition of folding and the high sensitivity of sterols to ultraviolet light add support to the finding made with absorption measurements that sterols are involved in the folding process.

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